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In Vitro Cytotoxicity Assays of Human Epidermal Keratinocytes in Culture Exposed to Sulfur Mustard

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13. ABSTRACT (*Maximum 200 words*)

A number of studies have described several *in vitro* cytotoxicity assays that are convenient methods for assessing cell viability. Viability assessment of human peripheral blood lymphocytes (PBL) by flow cytometry of propidium iodide (PI) exclusion has been a useful tool for screening candidate prophylactic (P) as well as therapeutic (T) drugs for medical countermeasures against sulfur mustard (HD) for many years at our institute. Our laboratory has reported problems associated with simple vital dye uptake as a measure of cytotoxicity in human epidermal keratinocytes (HEK) following *in vitro* exposure to HD. We have evaluated five alternative assays of cell viability for HEK exposed to HD *in vitro*: Calcein-AM, a fluorescent dye; Alamar Blue, a redox fluorescent dye; Neutral Red, a chromogenic dye; Methyl tetrazolium salt (MTS), a colorimetric assay; and viable cell number. At the highest HD dose tested, the viable cell number, Calcein-AM, Alamar Blue and Neutral Red assays were more sensitive than propidium iodide uptake. MTS failed to demonstrate cytotoxicity at any HD concentration tested. The best observed cytotoxic dose response was obtained with the viable cell number assay, which, although labor intensive and slow, is reliable and provides a concentration-effect curve for HD.

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Introduction

Sulfur mustard (2,2'-dichlorodiethyl sulfide, HD) is a powerful vesicating chemical warfare agent (CWA) that causes profound injuries to the skin, eyes, and lungs. It was used as a chemical weapon during World War I and the Iran-Iraq conflict and remains a significant military and civilian threat. Human peripheral blood lymphocytes (PBL) and human epidermal keratinocytes (HEK) have been used as *in vitro* models to study the cytotoxicity effects of HD (1,2) and to develop therapeutic regimens against this vesicant agent. However, problems have been detected with the uptake of dye, such as propidium iodide (PI), for a measure of cytotoxicity in HEK (3), since the process of trypsinization required to detach the HEK may influence dye uptake. The viability assays described in this technical report allow the cells to remain *in situ* in 24-well plates for the entire cytotoxicity assessment.

Five different assays of cell viability for HEK grown *in vitro* and then exposed to HD were evaluated: Calcein-AM, a vital fluorescent dye; Alamar Blue, a redox fluorescent dye; Neutral Red, a lysosomal chromogenic dye; Methyl tetrazolium salt (MTS), a metabolic colorimetric assay; and a viable cell number method. We describe these assays and compare our findings with those obtained using PI uptake as a cytotoxicity test in HEK.

Materials and Methods

Reagents

Human epidermal keratinocytes (HEK), keratinocyte growth media (KGM™), Trypsin/EDTA solution, and trypsin neutralizing solution (TNS) were acquired from Clonetics™, San Diego, CA, USA. RPMI-1640 (with and without phenol red as indicator), propidium iodide (PI) and Neutral Red (NR) vital dyes were obtained from Sigma, St. Louis, MO, USA. Sulfur mustard (HD; approximately 98% purity) was obtained from the U.S. Army Edgewood Chemical and Biological Center (ECBC), Aberdeen Proving Ground, MD, USA. Calcein-AM was obtained from Molecular Probes, Eugene, OR, USA. MTS (3-(4,5-dimethyl thiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) was acquired from Promega, Madison, WI, USA. Alamar Blue was obtained from AccuMed International, Inc., West Lake, OH, USA. Ethanol was obtained from Pharmco Products, Inc., Brookfield, CT, USA. Acetic acid was purchased from Fisher Scientific, Fair Lawn, NJ, USA. Twenty-four - well plates were obtained from Becton Dickinson & Company, Lincoln Park, NJ, USA. Coulter® Balanced Electrolyte Solution (Isoton II) was purchased from Coulter Corporation, Miami, FL, USA.

Cell Culture-Human Epidermal Keratinocytes (HEK)

HEK were grown in 24 multiwell plates (Falcon Primaria or Corning) and incubated at 37°C in a 5% CO_2 incubator (4). The percent of confluence was estimated by microscopy before the HEK were exposed to sulfur mustard. Typical confluencies determined were between 60 and 100%.

Sulfur Mustard (HD) Exposure of HEK

HD was diluted in KGM™ and added to the multiwell plates to yield final concentrations from 50-300 μM. After one hour at room temperature in a chemical surety hood, the multiwell plates were transferred to 37°C in a 5% CO_2 incubator for the duration of the postexposure incubation period.

Calcein-AM Analysis

Cell viability was assessed by using Calcein-AM as an indicator of membrane integrity. Viable cells take up Calcein-AM, and the acetoxymethyl groups are cleaved by intracellular esterases, thereby trapping the dye inside the cells, whereas the nonfluorescent dye leaks out of dead cells. The media from each well was removed by aspiration 24 hours after HD exposure. Immediately, 1 mL Calcein-AM (10 μg/mL) was added to each well in the dark to avoid degradation and decomposition of the dye. The multiple well plates were incubated for 1 hour at 37°C in a 5% CO_2 incubator. Media was removed by aspiration at the end of the incubation period to eliminate the nonabsorbed Calcein-AM, and 300 μL of RPMI-1640 (without phenol red) was added. The multiple well plates were analyzed using a Cytofluor Multi-well plate reader (PerSeptive Biosystems, Framingham, MA, USA) with excitation at 485 nm and emission measured at 530 nm.

Alamar Blue Analysis

Cell viability was determined by using Alamar Blue, a fluorescent dye, as an indicator of mitochondrial metabolism. Actively metabolizing cells cause a color change with Alamar Blue that is detected spectrofluorometrically. At 24 hours after HD exposure, the media in the plates were removed by aspiration and rinsed 3 times with 300 μL of RPMI-1640 (without phenol red). After the third rinse, 500 μL of Alamar Blue (5% Alamar Blue in RPMI-1640 without phenol red) was added to each well, and the plates were incubated at 37°C in a 5% CO_2 incubator for 1.5 hours. At the end of the incubation time, the plates were analyzed by reading them on a Cytofluor Multi-well plate reader with excitation at 530 nm and emission measured at 590 nm.

Neutral Red Analysis

Cell viability was determined using Neutral Red, a chromogenic dye, as an indicator of lysosomal activity. Live cells demonstrate a chromogenic change with Neutral Red that is detected spectrophotometrically. The medium in each well was removed by aspiration 24 hours after HD exposure. One mL of Neutral Red (50 μg/mL) was added to each well in the dark and incubated for one hour at 37°C in a 5% CO_2 incubator. The Neutral Red solution was aspirated and the wells were rinsed twice with 500 μL of RPMI-1640 (without phenol red). One mL of ethanolic extraction solution (50% ethanol/1% acetic acid) was added to each well and incubated for 30 minutes at 37°C in a 5% CO_2 incubator. After the incubation period, 200 μL of each well was transferred to a clean 96-well plate and analyzed by using a Power Wave 200 Microplate Scanning Spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) set at 550 nm.

MTS Analysis

Cell viability was determined using MTS as an indicator of mitochondrial dehydrogenase activity, which causes a chromogenic change with MTS that is detected spectrophotometrically at 490 nm. At 24 hours after HD exposure, 200 μL of MTS solution was added to each well as described in Technical Bulletin #169 provided by Promega in their kit. The multiwell plates were incubated at 37°C in a 5% CO_2 incubator for an additional two hours, and approximately 200 μL of each well was transferred to a clean 96-well plate. The 96-well plate was read using a Power Wave microplate scanning spectrophotometer set at 490 nm.

Propidium Iodide Analysis

Cell viability was determined using PI as an indicator of deteriorating membrane integrity. Living cells exclude PI although it penetrates the plasma membrane of dead cells, binds to cellular nucleic acids, and fluoresces. At 24 hours after HD exposure, 24-well plates were removed from the incubator, and the supernatant fluid from each well of the plate was transferred to a 15-mL conical test tube. One milliliter of Trypsin/EDTA solution was added to each well and incubated at 37 °C in a 5% CO_2 incubator for 10 minutes. After this incubation period, 1 mL of trypsin neutralizing solution (TNS) was immediately added to neutralize the enzyme, and the fluid from each well was transferred to 15-mL conical test tubes and mixed. The 24-well plates were observed under a microscope to ensure that no remaining HEK were attached to the wells. One milliliter of each sample (from a total of 3 mL in the 15 mL tubes) was added to a vial that contained 20 mL of Isoton II for cell counting. The remaining HEK left in the 15-mL conical test tubes were spun in the centrifuge for 8 minutes at 425 xg. The supernatant fluid was aspirated, and 300 μL of fresh KGM medium was immediately added to each tube containing cell pellets (HEK), resuspended by mixing, and then transferred to a 12 x 75-mm test tube for flow cytometry. A solution of PI (0.3 mg/mL) was prepared and 50 µL was added to each test tube and mixed. The test tubes were incubated at room temperature for 3 to 5 minutes and then analyzed on a flow cytometer (FACSort, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) using an argon laser at 488 nm.

The chemical structures of the molecular probes used in these studies are shown in Figure 1, except for Alamar Blue, which is a proprietary compound.

Viable cells number

At 24 hours after HD exposure, the 24-well plates were removed from the incubator, and the supernatant fluid from each well was transferred to a 15-mL conical centrifuge tube. One milliliter of Trypsin /EDTA solution was added to each well and incubated at 37°C in a 5% CO_2 incubator for 10 minutes. After the incubation period, 1 mL of trypsin neutralizing solution (TNS) was immediately added to neutralize the enzyme, and the fluid from each well was transferred to the respective 15-mL conical tube. The 24-well plates were observed under a microscope to ensure that no remaining HEK were attached to the wells. One mL of each sample was added to a vial containing 20 mL of Isoton II. The cell count was read on a Coulter® Multisizer II cell counter (Coulter Corporation, Scientific Instruments, Miami, FL, USA). One milliliter of each sample was analyzed for percentage of PI uptake by flow cytometry as described above. The number of viable cells per well is calculated by multiplying the cell yield times the percentage PI negative (i.e., % viable). Percent viability is a ratio between control cell viable number and treated cell viable number. Table 1 summarizes the specific endpoint of each of the molecular probes for measuring a particular biological activity.

Table I

Dye	Indicator of:
Calcein–AM	Membrane integrity
Alamar Blue	Mitochondrial metabolism
Neutral Red	Lysosomal activity
MTS	Mitochondrial dehydrogenase activity
Propidium Iodide	Membrane integrity

The dyes described above and their proposed indicator mechanism can be found in Haughland, RP, *Molecular Probes Handbook of Fluorescent Probes and Research Chemicals*, 5th Edition, 1992-1994, K.D. Larison, Ed., USA.

Results

A typical histogram obtained when HD-exposed HEK (50, 100 and 300 μM HD) were analyzed with *in vitro* assays for cytotoxicity using Calcein-AM, Alamar Blue, and PI is illustrated in Figure 2.

No apparent differences were observed between these fluorescent dyes at 50-100 μM HD. Viability decreased approximately 5-10% with these fluorogenic dyes. Therefore, there is a weak correlation of cell viability and dose-response using these fluorescent probes as indicators of HD cytotoxicity in HEK. A greater HD-induced cytotoxicity was correlated with 46-55% loss of viability at 300 μM of HD using the Calcein-AM and Alamar Blue dyes. However, PI showed only a 20% loss of viability at 300 μM HD. The reason for this difference at the higher dose of mustard is unclear at this time.

HEK viability was assayed with two different chromogenic dyes, NR and MTS. Addition of HD does not cause much cytotoxicity in HEK when measured by MTS even when tested at 300 μM (Figure 3).

The NR assay is also relatively insensitive, so the chromogenic assays do not provide reasonable assessments for HD cytotoxicity in HEK. Direct comparisons of the dye data with the viable cell number assay showed a generally lower sensitivity of these assays (Figure 4) in measuring cytotoxicity of HD in HEK. The viable cell number assay appeared to have the most linear response to HD.

Discussion

The purpose of this study was to compare cytotoxicity assays for assessing cell viability in HEK exposed to HD. The main features sought with these assays are ease of use, accuracy and rapid indication of toxicity. We wished to compare these rapid assays with propidium iodide uptake as measured by flow cytometry, the "gold standard" of previous cytotoxicity measurements in our laboratory. Propidium iodide (PI) is a fluorescent stain for nucleic acids and has shown good results using PBL since it fluoresces in the red and is taken up by dead cells exposed to HD. Flow cytometry gives a rapid and sensitive analysis of living and dead cells using this dye.

However, PI uptake in HEK is not as effective as the PI assays used with PBL. A possible explanation for this observed discrepancy between the two types of cells may be the enzymatic detachment procedure used to harvest HEK (trypsin/EDTA). Further studies are needed to elucidate this discrepancy.

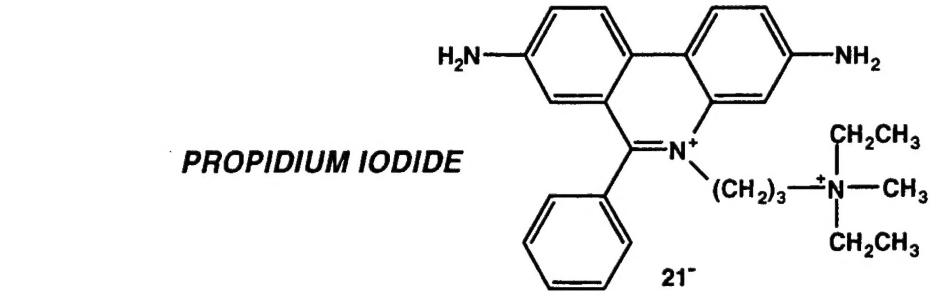
In terms of the specificity of the MTS assay, the results showed poor correlation between HEK cell viability and HD-dose response, although the procedure appears to work well using PBL. The reason for this result is not understood at present and will require much more research before it can be used.

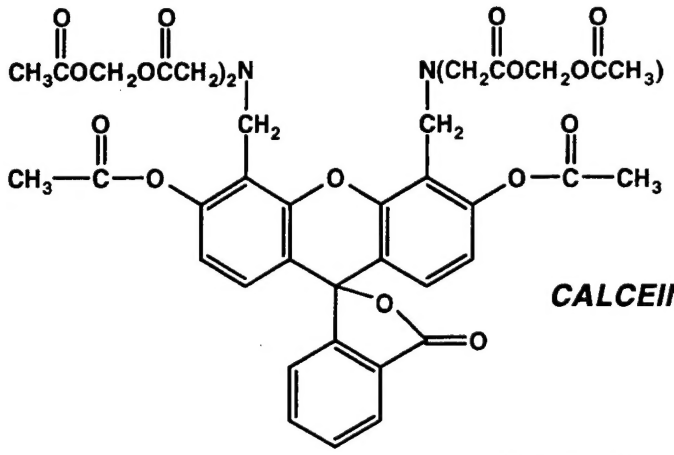
Calcein-AM, a nonfluorescent substance that is intracellularly converted to the green fluorescent calcein by esterase activity in viable cells, was used to stain HEK after HD exposure and 24 hours of incubation. This assay is based on the retention of the calcein marker in living cells and can be measured in an automated microplate fluorescence scanner, such as the Cytofluor II from Molecular Probes, using tissue culture plates with either a 24- or 96-well format. This assay showed a good correlation between percent viability and 300 μM HD when compared with the viable cell number assay. Alamar Blue is a nontoxic metabolic indicator that becomes fluorescent upon mitochondrial reduction in viable cells. It can be analyzed in a 24- or 96-well tissue culture plate in the Cytofluor, allowing the measurement of a high number of samples in a relatively short time. These two fluorescent assays have been reported to be extremely sensitive, economical, simple and nontoxic methods to evaluate cell cytotoxicity. However, for *in vitro* cytotoxicity assays in HEK exposed to HD, these assays appear to be less sensitive in comparison with the viable cell number assay and may be used in a qualitative but not a quantitative mode.

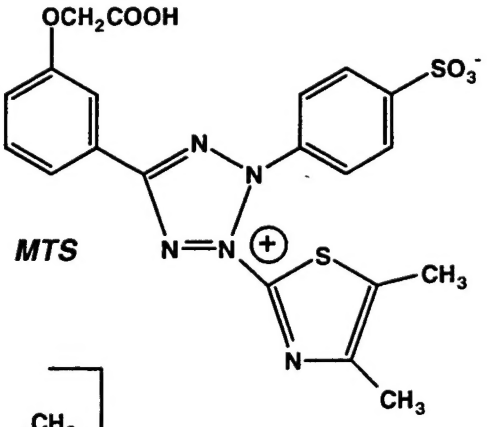
Of these six tested assays, the one assay that demonstrates a clear dose-effect relationship for HD-induced cytotoxicity is the viable cell number assay. Chromogenic and fluorogenic assays do not show this dose-dependent effect of HD, although they are more rapid and not as technically demanding as the flow cytometry method. However, flow cytometry requires detached cells, and extensive manipulation of cells such as HEK may compromise results. The viable cell number assay is attractive, since this assay has a few main features such as ease of use and accuracy for indication of HD-induced cytotoxicity, although it is slow and labor intensive.

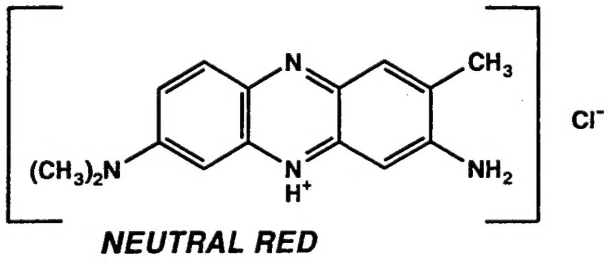
This study has also uncovered a few technical problems that must be considered in assay development. COSTAR-24 well plates purchased from Becton Dickinson & Company, Lincoln Park, NJ, USA, did not provide optimal conditions for growing HEK. After determining the optimal growing conditions for HEK using different multiwell plate suppliers, multiwell Falcon Primaria plates supplied by Becton Dickinson & Company, Lincoln Park, NJ, USA, were selected for routine growth of HEK. Other factors can affect HEK experiments: (a) passage number of HEK, (b) incubation time, (c) batch number, (d) percentage of confluency, (e) lot number of HEK, and (f) incubator vibration (5). All of these factors must be kept the same if valid comparisons between experiments are to be made.

In conclusion, this study has shown that the various chromogenic and fluorescent assays described above are not as accurate as the viable cell number assay and have no advantage to the researcher as a rapid method to determine cytotoxicity to HD.









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Structure Unavailable
Proprietary Information

Figure 1. Chemical structures of the available molecular probes used in this study

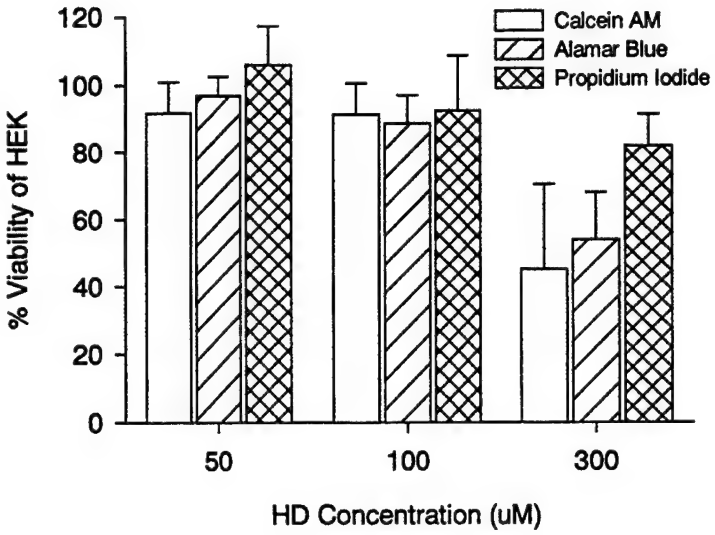

Figure 2. Percent of cell viability response of HD-exposed HEK using two different fluorescent probes as indicated in the figure caption. The cell viability response is compared with the propidium iodide assay. The data presented are means ± s.d. of at least six different trials in several representative experiments.

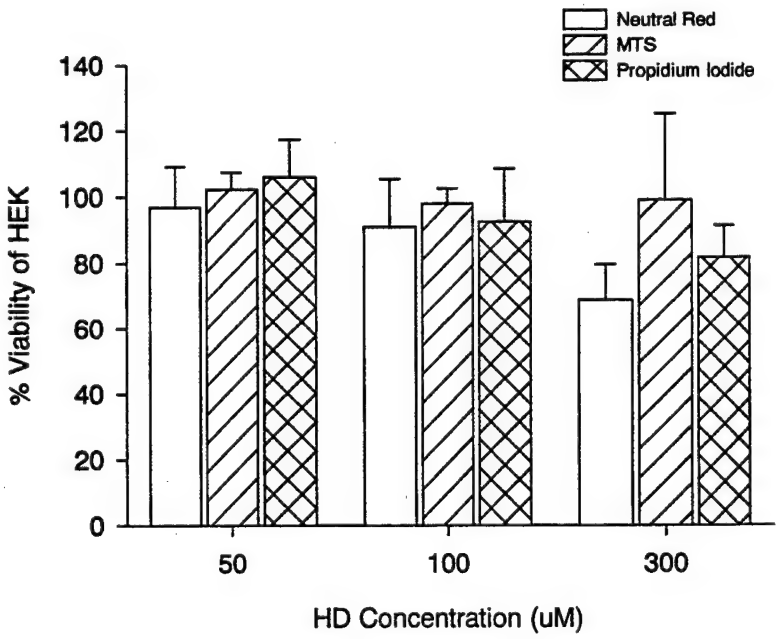

Figure 3. Percent of cell viability response of HD-exposed HEK using two chromogenic dyes. These results are compared with the well-established cell viability method, PI uptake. The illustrated data are means ± s.d. of at least six determinations in different representative experimental trials.

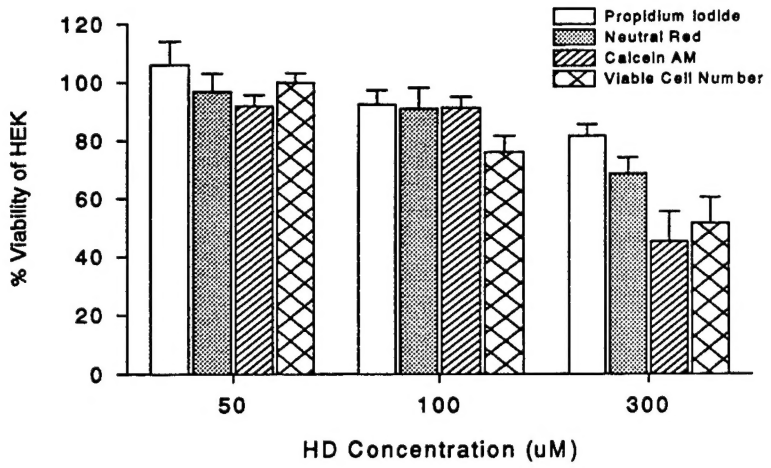

Figure 4. Percent of cell viability response of HD-exposed HEK using fluorescent and chromogenic probes. These results are compared with the viable cell number assay. The data shown here are the means ± s.d. of at least six determinations in different representative experimental trials.

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